

1 INTRODUCTION

Malignant neoplasm represents a major cause of morbidity and mortality all over the world. Cancer is one of the main causes of death after infection and heart diseases in all societies (Kwok, 2001) and is the fatal leading disease in the world (Kwok, 2001). Malignant lymphoma is a diverse group of cancers. This cancer usually arises in lymph tissues such as lymph nodes (lymph glands), spleen, and bone marrow.

Non-Hodgkin's lymphoma (NHL) begins when a lymphocyte (B cell or T cell) becomes abnormal. The abnormal cell divides to make copies of itself. The abnormal cells are cancer cells. They do not die when they should. They do not protect the body from infections or other diseases. Also, the cancer cells can spread to nearly any other part of the body (Moslehi et al., 2006).

NHL consists of many distinct disease entities. In the World Health Organization (WHO) classification, NHLs are categorized according to morphological, immunophenotypical and molecular features into B-cell neoplasm, T-cell and natural killer (NK) cell neoplasms (Harris et al., 2000). The WHO classification is the latest classification of lymphoma. It was based on the "Revised European- American Lymphoma classification" (REAL) (Shen *et al.*, 2007)

Hepatocellular carcinoma, multiple myeloma, and non-Hodgkin's lymphoma (NHL) are the three most frequent tumors that have considerable mortality increases since 1995 (Howell and Rose-Zerilli, 2007)

As reported by the World Health Organization International Agency for Research on Cancer (IARC) NHL rate is growing worldwide and is more in developed countries (Müller et al., 2005).

Although NHL is known to be influenced by immunosuppression and viral infections agents, there are several significant reasons that show NHL can be affected by some genetic component as well. Environmental agents and inherited genetic abnormalities may result in creation of permanent chromosomal variations that underlie NHL. Studies on genetic variation of cytokine genes can help to understand how NHL begins and lead to identifying successful treatments. One of these important genetic variations are single nucleotide polymorphisms (SNPs) which is one of the most common occurrences and is distributed across the entire cytokine genes such as interleukin-10 gene.

Cytokines are hormone like, small proteins, peptides, or glycoproteins molecules secreted by some cells of the nervous system or immune system (Howell and Rose-Zerilli, 2007). Cytokines act as regulators on target cells within the hematopoietic mechanisms such as immune response, inflammation and tissue repair so variation in their amount and their

structure can contribute in the disease process. It has been reported that several common tumors produce inflammatory cytokines therefore cytokines may have a role in progression of some cancers (Havranek et al., 2005). Cytokines, antitumor function may be via T-helper cell type 1(Th1) in response to supporting cell mediated immunity or a T-helper cell type 2 (Th1) in response to supporting humoral immunity (Purdue et al., 2006).

Interleukin-10 (IL10) is an important immunoregulatory cytokine that is involved in the immune response of both infectious and autoimmune diseases. IL10 has an anti-inflammatory, regulatory effect on immune responses by inhibiting the production of immunoactive molecules, such as tumour necrosis factor α (TNF- α) in T cells and monocytes. It can also have an inflammatory function by stimulating B cells to produce immunoglobulins and express MHC (major histocompatibility complex) class II (Cervenak et al., 2000). It has been assumed that IL10 affect pathogenesis of non-Hodgkin lymphoma through some of its biological activities which are regulation of balance between T-helper 1, and T-helper 2 cells, immune responses and control of lymphoid cell differentiation (Lee et al., 2005).

Some of the polymorphisms on the promotor region of the gene contribute to IL10 secretion. -1082 G, -819 C alleles are associated with higher IL10 secretion while -1082 A, -819 T alleles are associated with lower IL10 secretion (Bagnoli et al., 2007). Therefore these SNPs can be associated to the characteristics of IL10 protein like antitumor responses or

tumor progression (Havranek et al., 2005). This study was carried out using PCR- Restriction Fragment Length Polymorphism (PCR-RFLP) genotyping on *IL10* gene. The DNA samples were extracted from cases and control subjects. Then, the association between the *IL10*-1082 G/A and *IL10*-819 C/T polymorphisms and development of NHL in three ethnic groups in Malaysian was investigated. The findings of such a study on the Malaysian population would also provide a database for future assessment of treatment for those having NHL.

2 Objective

To study the association between SNPs in interleukin-10 (*IL10*-1082 G/A and *IL10*-819 C/T) and non Hodgkin lymphoma (NHL) patients from three major ethnic (Malays, Chines, Indian) in Malaysia.

3 LITERATURE REVIEW

3.1 *Cancer*

The tendency of cancer rate and mortality are increasing annually. The International Agency for Research on Cancer (IARC) conducted a broad investigation on cancer in 10 countries and geographical regions. The results reported that of the 7.6 million new cancer cases diagnosed worldwide annually, more than half are contributed in developing nations (Twombly, 2005). Likewise, in terms of number of new cancer cases, Parkin et al. (2005) estimated that there were 10.6 million new cases, 6.7 million deaths, and 24.6 million persons living with cancer (within 5 years of diagnosis) in 2002 (Parkin et al., 2001).

Changes in genes structure and the combination of genetic alternations are responsible for coding different types of essential vital proteins. Alternations, such as regulating gene expression variations, cell division, cell differentiation, cell death and tissue of origin may cause different types of cancer (Almadori et al., 2004, Warnakulasuriya et al., 1999). The terms which express the development of cancer are carcinogenesis, oncogenesis or tumorigenesis (Grizzi and Chiriva-Internati, 2006). Carcinogenesis consists of 3 stages: Initiating event through which permanent mutation occurs in gene; promotion in which the cells divide; and progression, which refers to the development of over time by mutation in, divided cells.

Genetic susceptibility, environmental risk factors and inherited genomic changes of cancer gene are all involved in carcinogenesis, but the response of cells to the risk factors of environmental agents is different (Nasca, 2001).

Although there are so many acquisitive risk factors associated with smoking, drinking or tobacco chewing, intrinsic factors is required for cancer to occur. Most of the external risk factors need to convert to biological active forms to be able to have effect on the host DNA. In addition to acquisitive factors, there are hereditary factors, which are activated through the process of deletion of a single gene or mutation. Such a process is often followed by Mendelian transmission. However, as the gene mutation is rare, it just plays a small role in cancer incidence (Park et al., 2000, Nasca, 2001).

3.2 Lymphoma

The lymphatic system is a network of vessels that helps filtering out infection and disease by white blood cells and antibodies produced there in. The cancer of this system is named lymphoma. It happens when lymphocytes are dividing without control and create an extra mass of tissue that can turn into a tumor. They often originally start from only one lymph node group or one extra nodal site such as skin, thyroid, lung, nasopharynx, tonsil, breast, and bone. The three major types of lymphocytes are B-cells, T-cells, and natural killer cells. Lymphomas are the malignant counterpart of these cells. The symptoms of early

appearance of malignant lymphoma is a progressive, usually painless, enlargement of a single lymph node or lymph node group in an otherwise asymptomatic patient. Lymphoma is commonly divided in two main group Hodgkin's lymphoma (HL) and non-Hodgkin's lymphoma (NHL) (Moslehi et al., 2006).

According to Chai et al. (1999) for the period between 1981 to 1983, lymphoma was one of the top10 cancers in East Malaysia (Chai et al., 1999). They also reported the range of lymphoma in East Malaysia is rather similar to West Malaysia, where the HL: NHL ratio is 1:9 and also 4.5% and 3.5% of cancers in Sarawak and Sabah respectively are lymphoma. The pattern of lymphoma in Sabah and Sarawak were moderately similar, except for a very low occurrence of T-NHL in Sarawak (3.3%) (Chai et al., 1999). These differences could be due to the genetic factors, because both groups were living in comparable environment.

3.3 Non Hodgkin Lymphoma (NHL)

The diagnosis of non-Hodgkin lymphoma (NHL) evolved from Thomas Hodgkin's paper entitled 'On some morbid appearances of the absorbent glands and spleen' (Lauder et al., 1984).

NHL is largely divided in two major groups: B-cell lymphoma (which develop from abnormal B-lymphocytes, and is the most common), and T-cell lymphomas (which develop

from abnormal T-lymphocytes). B-cells develop into plasma cells that produce antibodies to fight infection, while T-cells attack foreign invaders (bacteria, viruses, cells) directly.

NHL can begin in a particular lymphatic organ such as the spleen, or in lymph tissue in organs like the stomach or intestines. Abnormal lymphocytes can get to any part of the body because lymphocytes can move to all parts of the body, all the way through the lymphatic vessels and blood stream. Thus, NHL can start in or spread to any part of the body (Harris et al., 2000).

The most recent system for classification of NHL is the REAL/WHO (World Health Organization) classification. This method classifies lymphomas based on how they look under a microscope, clinical features, race, geographic distribution, microbiologic features, and the chromosome features of the lymphoma cells. In another classification, NHL can be divided into low grade NHL such as small cell lymphocytic (or chronic lymphocytic leukaemia), splenic marginal zone lymphoma, mantle cell, malt lymphoma, follicular, and high grade NHL like diffuse large B cell, anaplastic large cell lymphoma, diffuse mixed cell lymphoma, Burkitt's lymphoma (Dreier et al., 2003).

In the 1980s, the lymphoid origin of NHL was confirmed at the molecular level with the identification of specific immunoglobulin (Ig) gene and T cell receptor (TCR) gene rearrangements in B and T cell lymphomas, respectively (Berinstein et al., 1993).

3.3.1 NHL Causes

The reasons of NHL remain unknown, but differences in frequency rate and various sub-types of lymphoma among different populations lead to the assumption that environmental factors such as carcinogens, pesticides, herbicides, bacterial and viruses infectious like Epstein-Barr, human T-lymphotropic virus type 1 (HTLV-1), HIV, hepatitis C or certain bacteria, such as H-pylori play a role. The risk of NHL is more probable in people taking immunosuppressant drugs after an organ transplant those infected with autoimmune disease such as rheumatoid arthritis or psoriasis or AIDS people with a diet high in meats and fat and those who have had past treatment for Hodgkin's lymphoma. In addition it has been seen the risk of NHL is higher in individual with a family history of NHL (Fadilah, 2009).

3.3.2 Symptoms of NHL

The most common signs of NHL may include, chills, swollen and painless lymph nodes, fever, night sweats, unexplained weight loss, lack of energy and feeling very tired, itching, coughing, trouble breathing or chest pain and, pain or swelling of the abdomen (Fadilah, 2009).

3.3.3 Treatments of NHL

Treatment of NHL depends on the type of NHL, the patient's age, stage, if it is indolent or aggressive. The most common treatment comprises: Chemotherapy, biological

therapy, or radiation therapy.

Stem cell transplantation that is recommended to those who relapse from the disease after initial treatment. For some patients transplantation may not be a suitable treatment, due to either older age or medical problems.

Indolent lymphomas, such as follicular lymphoma have high level of relapse. Although chemotherapy alone has not yet caused an improvement in overall survival, the diagnosis is still very good, and patients might live for 20 years or more.

Approximately thirty percent to sixty percent of patients with “aggressive” NHL can be cured and standard of treatment attains long-term remission in less than half of NHL cases (DeNardo, 2005, Fadilah, 2009).

3.3.4 Incidence of NHL in the world

The data from World Health Organization International Agencies regarding cancer research revealed the high rise of NHL in the world and the highest rate of this cancer is in developed countries (Parkin et al., 2001). Development of NHL in western countries is prevalent in both male and female, black and white and noticeably among older peoples (Müller et al., 2005).

Increased incidence of non- Hodgkin lymphoma and the high mortality rate caused due to this cancer make it the fifth most common cancer after breast, lung, prostate and colon cancers in the United State (Groves et al., 2000). The highest rate of NHL cancer was reported among white men in United States (Newton et al., 1997).

More than 40,000 new cases per year will be diagnosed in the United State (Boring et al., 1991), and the incidence is increasing because of the development of NHL in patients with the acquired immunodeficiency syndrome (AIDS) (Devesa et al., 1987, DEVITA et al., 1970).

NHL has low incidence in Eastern Asian countries, Southern Africa, tropical Africa. The North Africa has higher rate compared to the other parts of this continent (Cook-Mozaffari et al., 1998, Sukpanichnant et al., 1998)

Although epidemiologic studies are flawed by methodology weakness, some reports associated a higher risk of NHL in farmers using herbicides, particularly those with phenoxyacetic acid (Levi et al., 1989, Wigle et al., 2002).

3.3.5 Incidence of NHL in Malaysia

Peh et al. (2001) showed that in Malaysia high-grade diffuse lymphoma is the dominant subtype of NHL but there is low prevalence of follicular lymphoma. They also reported a very low incidence of T-NHL in Sarawak. According to this study, among the races in Sarawak, Chinese are less susceptible in developing NHL and the occurrence of EBV-

associated T/NK lymphoma in the Chinese population of Sarawak is very low (Peh et al., 2001). In another study they also announced that Burkett's lymphoma is 37.0% of childhood NHL and about 5.0% of adult NHL in Malaysia (Peh et al., 2002).

3.4 Single Nucleotide polymorphisms (SNPs)

A number of studies has been done on single nucleotide polymorphisms SNPs, because of their great influence on human phenotype.

They are the most abundant form of genetic variation in humans. SNPs are kind of DNA sequence changes resulting from mutation. This type of polymorphism is one of the most common occurrences with the incidence frequency of about 1 in 1000 base pairs (Shastry, 2002).

Almost all common SNPs have only two alleles (Brookes, 1999, Shastry, 2002). Based on this matter whether the encoded amino acid is changed or not, SNPs are categorized into two groups of synonymous (coded amino acids without any change) and non-synonymous (coded amino acids with change). Non-synonymous SNPs are divided into missense (change results in a different amino acid) and nonsense (change results in a premature stop codon) (Shastry, 2002).

There is low or even no effect as a result of presence of SNPs in the genome. SNPs can use as genetic markers, for example to detect the genes association with a disease (Brookes, 1999, Shastry, 2002).

Frequency of polymorphisms is influenced by variation of ethnicity of a population in different parts of the world or even within the same country, environmental effects, and other factors (Hunter, 2006). Knowledge of the frequency of polymorphisms in a population could therefore be important for planning of health care in future.

According to Shastry (2002), comparative studies on identical twins and fraternal twins showed that SNP is one of the factors associated with susceptibility to many common diseases as well as every human traits such as tallness and curly hair (Shastry, 2002). This type of knowledge may in the future provide useful information before disease diagnosis, or offers personalized treatment after disease diagnosis.

In pharmacogenomic research SNPs play a great role to drug production from target identification to clinical trials and are useful to understand the response of individuals to the drug (Kwok, 2001).

3.5 IL10 *protein characteristic*

T helper cell activation promotes differentiation of naive Th0 cells into Th1 and Th2 cells. Th1 cells produce interleukin 2 (IL-2), interferon (IFN)-gamma, tumour necrosis factor (TNF), while Th2 cells produce IL-4, IL-5, IL-10 and IL-13. Therefore cytokines are classified into two types: Th1 (proinflammatory) cytokines and Th2 (anti-inflammatory) cytokines. Interleukin 10 (IL-10) is a pleiotropic and Th2 cytokine with potent immunoregulatory activities against both infectious and autoimmune diseases (Yilmaz et al., 2005, Torkildsen et al., 2005). It is a non-covalent homodimer with 178 amino-acids of two polypeptide chains of 36 kDa (Yilmaz et al., 2005). It has been suggested that a prominent anti-inflammatory property for this cytokine through direct influence over production and function of a number of pro-inflammatory cytokine (Mocellin et al., 2004). It inhibits NF_κB (Nuclear Factor-κB), a transcription factor involved in the production of inflammatory cytokines like IL-1, IL-6, IL-8, and tumor necrosis factor while stimulating the production of cytokine inhibitors like IL-1 receptor antagonist and the soluble tumor necrosis factor receptor by monocytes/macrophages and neutrophils (Im et al., 2004). Furthermore its ability in suppression of T cell activity in immune response and prevention of tumor antigen presentation to CD8₊ cytotoxic T lymphocytes through inhibiting expression of major histocompatibility complex (MHC) class I and II antigens, indicate its contribution to tumour-related immunosuppression (Giordani et al., 2003, Fife et al., 2006). Im et al. (2004) showed that severe chronic enterocolitis (inflammatory bowel disease) progress in IL-10-deficient mice that support its anti-

inflammatory role. However it acts to have a positive inflammatory function by activating polyclonal B cells and thus supporting the production of autoantibodies. Im et al. (2004) also showed that anti-IL10 is a good treatment in systemic lupus erythematosus (SLE) patients. Under IL-2 deficiency, IL10 inhibits apoptosis of T cells and supports the expansion of T-cell clones (Taga et al., 1993, Pawelec et al., 1995). IL10 has been suggested to have contribution on differentiation of some other immune cells such as mast cells, granulocytes, dendritic cells, keratinocytes, endothelial, natural killer cells, antigen-presenting cells (Eskdale et al., 1999, Im et al., 2004, Persico et al., 2006, Lee et al., 2005). Activated CD4⁺ T cells, NK cells, monocytes, lymphocytes, B cells, thymocytes, macrophages normally secrete IL10, and dendritic cells (Eskdale et al., 1999, Mocellin et al., 2004, Domingo-Domenech et al., 2007). Expression of IL10 can be regulated by transcription factors Sp1 and Sp3 at transcription level; these factors are expressed by several cell types (Tone et al., 2000, Moore et al., 1993, Mocellin et al., 2004). On the other hand IL10 has negative effect on its production somehow by suppressing IL10 producing Tr 1(Treg) cells (Yilmaz et al., 2005).

3.6 *IL-10* gene

The *IL-10* gene is highly polymorphic and encodes 5 exons, spans; 5.2 kbp. The human *IL10* gene is located on chromosome 1 (1q31–q32) (Matsumoto et al., 2003, Yu et al., 2004). A number of studies have shown that, there are at least 49 disease-associated polymorphisms in the IL10 gene. These 49 polymorphisms consist of 46 SNP, 2

microsatellite polymorphisms, and 1 small (3bp) deletion (Gibson et al., 2001). Twenty-eight of these polymorphisms arise in the promoter region, 20 polymorphisms are noncoding intronic or synonymous substitutions and 1 polymorphism cause a change in amino acid sequence (Persico et al., 2006). Promoter of *IL10* includes region of at least 5 kb upstream of the transcription start point (Yao et al., 2008a). The promoter has two 2 CA-repeat microsatellites loci, IL10.R and IL10.G, 4 kb and 1.1 kb alternatively and also number of single nucleotide polymorphisms (MacKay et al., 2003). Some of these SNPs are in proximal region and some are in distal region of the promoter. Those in proximal region such as *IL10*-1082 G/A (rs1800870) lies between -4kb and -1.1 kb, *IL10*-1087 G/A (rs1800896) localized in a putative Ets (E-twenty-six specific) transcription factor binding site, *IL10*-824 C/T (rs1800871) is within a putative positive regulatory region and the *IL10*-597 C/A (rs1800872) located within a putative STAT-3 binding site and a negative regulatory region (Hee et al., 2007). *IL10*-819 C/T (rs1800871) and *IL10*-592 C/A (rs1800872) are in proximal region as well. *IL10*-3575 A/T (rs1800890) and *IL10*-2763 C/A (rs6693899) are examples of distal SNPs, which are between -1.3 kb and -4.0 kb (Yilmaz et al., 2005, Gibson et al., 2001, Eskdale et al., 1999).

3.7 Heritable production of IL10 protein and polymorphisms

Since stimulation of whole blood cultures *in vitro*, from first-degree family members with bacterial lipopolysaccharide (LPS) resulted in noticeable differences between individuals

in secretion of IL10, therefore differences in IL10 production might be associated with hereditary component (Eskdale et al., 1999, Fife et al., 2006). It has been reported that Inter-individual variations in IL10 production can be related to microsatellites and SNPs in promoter region of IL10 gene through changing transcription rate (W.Zammiti, 2006, Yao et al., 2008a). Among proximal SNPs -1082 G, *IL10*-819 C and -592 C alleles are associated with higher IL-10 secretion while -1082 A, -819 T and -592 A alleles are associated with lower IL10 secretion (Bagnoli et al., 2007). In distal region there is a tendency for a higher IL10 production for A allele of both *IL10*-3575 A/T and *IL10*-2763 C/A SNPs (Yilmaz et al., 2005). Furthermore it has been demonstrated that IL10.R and IL10.G microsatellite haplotypes are related to different level of IL10 expression *in vitro* while microsatellite IL10.R2 is associated with increased IL10 production and IL10.R3 with reduced production (MacKay et al., 2003). Turner and colleagues (1998) also showed that the difference in IL10 production is related to the presence or absence of an 'A' at position *IL10*-1082 G/A while the homozygous AA form of this allele causes low IL10 protein expression but GG homozygous is linked to the high IL10 expression (Havranek et al., 2005, Fife et al., 2006). However, the precise role of polymorphisms engaged in alteration production of this protein is still unclear (Lech-Maranda et al., 2004, Howell and Rose-Zerilli, 2007).

3.8 IL10 (gene/protein) is associated with disease

The wide range of IL10 functions in the immune system suggest that this cytokine has a significant role in a number of human disease states such as inflammation, autoimmunity, and transplant rejection. A positive linkage of *IL10* promoter haplotypes and SNPs to disease susceptibility and progression, or both have been reported (Yao et al., 2008a). Oral *et al.* (2006) demonstrated that decrease in the frequency of *IL10*-1082 A allele was associated with the progression of lung Tuberculosis (TB) and *IL10*-1082 AA genotype was demonstrated to be associated with TB pleurisy (Oral et al., 2006). Several studies have shown relations between *IL10* polymorphisms and the risk of and clinical outcome of some diseases like asthma (Torkildsen et al., 2005), systemic lupus erythematosus (Rothman et al., 2006), Epstein-Barr virus infections (Reuss et al., 2002), type 1 diabetes mellitus (Yilmaz et al., 2005) and multiple sclerosis (MS). IL10 prevents cytokine production and proliferation of CD4⁺ T cells and has a stimulatory influence on CD8⁺ T cells. This property could affect the occurrence and severity of supposed T-cell-mediated diseases such as MS (Torkildsen et al., 2005). Indeed, high levels of IL10 have been shown to be important for disease remission in MS (multiple sclerosis) because auto reactive T cells are generated in MS. In addition high IL10 production appears to be involved in pathogenesis of autoimmune diseases such as systemic lupus erythematosus and Rheumatoid Arthritis (RA) (Lech-Maranda et al., 2004, Morton et al., 2006)

Furthermore, elevated IL10 serum levels have been detected in several malignancies such as malignant melanoma, pancreatic and gastric adenocarcinoma (Morton et al., 2006, Clerici et al., 1998) and breast cancer (Mok et al., 1998, Kozowski et al., 2003). In breast cancer in addition to increased IL10 production, the factors that affect IL10 production like Th2 cells in peripheral blood, tumor microenvironment of patients (Liyanage et al., 2002), numbers of IL10 generating mononuclear cells (Merendino et al., 1999) and *IL10* mRNA (Venetsanakos et al., 1997) are also increased. Therefore, it can be concluded that high IL10 production may have a potent role in the progressing of breast cancer. However Giordani *et al.* (2003) described an increase in -1082 AA genotypes frequency (low *IL10* producer genotype) in breast cancer patients versus those in controls.

3.9 *IL10 protein and cancer*

Since low IL10 expression is a risk factor for disease or disease progression in some cancers, and high IL10 expression is a risk factor in others therefore level of IL10 can be associated with several cancers (Yao et al., 2008a). Advanced cutaneous malignant melanoma, breast cancer and prostate cancer has been associated with low IL10 level while cervical cancer, gastric, colon and renal cell cancers have been linked to excessive level of IL10. This cytokine has both immunosuppressive and anti-angiogenic functions, therefore it has consequently both tumor-promoting and tumor-inhibiting properties (Yao et al., 2008a).

Furthermore as many tumors and tumor-infiltrating lymphocytes express IL10, it has been suggested that IL10 can develop some cancers by mediating tumor escape from the immune response (Giordani et al., 2003). IL10 inhibits the tumoricidal capacity of macrophages, the cytotoxicity and cytokine production by tumor specific T cells and also blocks the tumor antigen presentation to CD8₊ cytotoxic T lymphocytes through suppressing expression of MHC class I and II antigens and decrease immune response toward transformed cells (Capei et al., 2003). IL10 promotes growth and differentiation of B cell tumors, and lead to angiogenic factor gene expression. Additionally a relation between IL10 and vascular endothelial growth factor expression has been proved in esophageal cancer (Yao et al., 2008a). However the *in vivo* study's result was opposite. When Chinese hamster ovary (CHO) cells, mammary adenocarcinoma cells and melanoma cells transfected with IL10, they were less effective at producing primary tumors or metastasis than untransfected cells in syngeneic and SCID (Severe Combined Immunodeficiency) mice.

Although the antitumor properties of this protein are not clear well, natural killer (NK) cells, T cells macrophages, and nitric oxide (NO) has been suggested to be implicated in this process. Furthermore, usage of IL10 has inhibited tumor metastasis and stimulated antitumor immune responses in various murine models. Some studies have revealed that IL10 inhibit dendritic cell function, which may affect the induction of a response against tumor cells (Havranek et al., 2005). It has been demonstrated that decrease of neovascularization in IL10 expressing tumors, therefore IL10 shows its antitumor and metastatic activities by

inhibiting angiogenesis *in vivo*. Some studies have demonstrated that the stimulation of tissue inhibitors of metalloproteases (TIMP) and inhibition of matrix metalloproteases (MMPs) secretion, induced by IL10 in prostate tumor cells, cause induction of angiogenesis *in vitro* (Cervenak et al., 2000). Some studies showed that the influence of IL10 produced by neoplastic B lymphocytes in the pathogenesis of lymphoid disorders which act as an autocrine growth factor which cause up-regulation of bcl-2 expression in some B-cell malignancies (Altieri et al., 2005). Production of macrophage proinflammatory cytokines is regulated with IL10. Low expression of IL10 influence on less efficiently suppression of proinflammatory cytokine production and so could increase risk of non-Hodgkin lymphoma (Rothman et al., 2006).

There is an association between the increasing level of IL-10 in serum and shorter survival of the patients with NHL and Hodgkin lymphoma (Blay et al., 1993, Hofmann et al., 2002).

Various cytokine productions have been linked to polymorphisms in the promoters region of the cytokine genes. Therefore, genetic alterations may directly and indirectly control carcinogenesis directly by influencing oncogenes and indirectly by varying cytokine expression (Tavtigian et al., 2001, McCarron et al., 2002).

There is a suggestion that occurrence of genetic polymorphisms in *IL10* genes are associated with an increased risk of non-Hodgkin lymphoma (NHL). The frequency of *IL10*-1082G allele was higher in patients with lymphoma especially for diffuse large B-cell lymphoma (DLBCL). *IL10*-1082 G allele was associated with longer freedom from progression (FFP) and overall survival (OS) (Blay et al., 1993, Altieri et al., 2005)

The -3575A and -1082G *IL10* polymorphisms are associated with an increased risk of DLBCL high *IL10*-expressing genotype (*IL10*-592 CC) is associated with increased risk of AIDS-associated NHL (Lech-Maranda et al., 2004)

Another research in diffuse large B-cell lymphoma (one form of NHL) has suggested that *IL10*-1082 G (high expression) allele not only can be a risk factor for NHL susceptibility at a marginal level of significance, but also it can improve NHL patients. This finding can show function of *IL10* as a cancer-promoting and cancer-inhibiting cytokine (Michaud et al., 2006, Howell and Rose-Zerilli, 2007).

Additional work is needed to clarify the genetic and biologic basis for *IL10* and lymphoma relationships.

3.10 SNP genotyping methods

With refer to the great effect of SNPs on human health; SNP genotyping has gone under consideration to develop methods for allele discrimination and signal detection, which are the two components of SNP genotyping (Kwok, 2001).

3.10.1 Allele discrimination methods

Allele discrimination methods include three methods of hybridization/annealing, primer extension, and enzyme cleavage(Kwok, 2001).

3.10.1.1 Allele-Specific Hybridization

This is the simplest method, which uses allele-specificoligonucleotide (ASO) probes that can be used in both label and non-label pairs. Three types of these methods are TaqMan: that in need of additional enzymatic process to detect the signals besides the allele-specific hybridization for the primary discriminating reaction. The advantage of Taqman assay is that sample processing can be reduced as the PCR amplification and genotyping assay are incorporated into a single step. Allele-specific polymerase chain reaction (PCR) is done by allele-specific primer annealing and allele-specific ligation. It needs DNA ligase to join two adjusted oligonucleotides.

3.10.1.2 Allele-Specific Single-Base Primer Extension

Allele-Specific Single-Base Primer Extension methods needs perfect annealing of designed primers to one nucleotide upstream of the polymorphic site. There are greater diversity of labeling strategies to make this method more adoptable compare to hybridization/annealing assays(Kwok, 2001).

3.10.1.3 Allele-Specific Enzymatic Cleavage

The analysis of restriction fragment length polymorphism (RFLP) of different variation in homologous DNA sequences is an important process in genome mapping and genetic disease analysis, which works on the base of allele-specific enzymatic cleavage. When an SNP happened at a restriction endonuclease recognition sequence an RFLP is generated. Multi-locus probe (MLP) paradigms and single-locusprobe (SLP) which is more sensitive compared to the MPL are two types of subdivided of RFLP analysis (Saiki et al., 1992).

Restriction enzymes are used to digest the DNA sample in RFLP analysis technique and the separation is done according to their lengths by gel electrophoresis. RFLP analysis technique is an inexpensive method besides genetic fingerprinting and plays a vital role in genome mapping, determination of risk for disease, localization of genes for genetic disorders and to detect polymorphisms (Groves et al., 2000).

3.10.2 Signal Detection Method

3.10.2.1 Mass Spectrometry

Detection of SNPs is based on different molecular weights of small DNA fragments rather than the labels' behavior. This method is done by matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) analysis. Alternative alleles can be discriminated in DNA fragments with length of 3–20 nucleotides (Kwok, 2001).

4 Materials and Methods

The entire buffer and chemical preparation were described in details in Appendix A.

4.1 *Study Design*

This is a case-control study to determine the association of -1082 and -819 SNPs in interleukin-10 gene with non-Hodgkin lymphoma in three major ethnic groups in Malaysia. The study protocol was approved by UMMC Ethnic Committee of Ministry Center, Medical Research and Ethnic Committee of ministry of Health (MOH) and also registered under National Medical Research Register (NMRR).

There were 320 NHL cases, 285 healthy controls for *IL10*-1082 G/A SNP and 312 NHL cases, 284 healthy controls for *IL10*-819 C/T SNP in this study. Patient samples which include 3 major ethnic populations in Malaysia were collected from UMMC and Ampang Hospital. These hospitals function as main referral center for hematologic diseases in Malaysia. Furthermore, healthy volunteers' samples were collected around the Klang Valley, an area in Malaysia including Kuala Lumpur and its suburbs, and adjoining towns in the state of Selangor. Malaysians from other states migrate to these areas because of rapid development and industrialization in these regions, therefore the collected samples are a good

representation of the Malaysian population since the members for both patients and healthy volunteers originated from different states in Malaysia.

4.2 DNA Extraction

Most (60%) of the study subjects DNAs were provided and the remaining 40 % were extracted from collected salivary swab or blood samples, using phenol-chloroform method.

4.2.1 Blood DNA Extraction

Day 1 extraction

Frozen blood samples of volume around 2-4 ml were thawed before transferring into Falcon tubes. About 8-10 ml of 1X red cell lysis buffer, depending on the volume of blood, was added to each tube. The mixture was mixed and spun at 3,500 rpm, temperature 10⁰C for 10 minutes. The supernatant was discarded, leaving the pellet. The pellet was resuspended in 4 ml of 1X red cell lysis buffer, by vigorous shaking until no clumps of cells were present. The suspension was spun again at 3,500 rpm, temperature 10⁰c for 10 minutes. The supernatant was discarded. Repeated washing with 1x red cell lysis buffer was done until the pellet appeared white. The Falcon tubes were inverted to drain off excess fluid. The pellet was resuspended in 80 µl of 10x proteinase K buffer, 400 µl of sterilized distilled water, 40 µl of

20% SDS, and 20 μ l of 20 mg/ml proteinase K. The mixture was incubated in 37⁰C waterbath overnight.

Day 2 extraction

The incubated suspension was cooled to room temperature on the following day. Next, 200 μ l of 6M NaCl was added, the mixture was subjected to pulse vortex. The suspension was then transferred equally into 1.5 ml microcentrifuge tubes, with volume of about 350 μ l in each tube. Phenol-chloroform (800 μ l) was added and the mixture was shaken until milky emulsion was formed. The emulsion was spun at 13,000 rpm, temperature 10⁰C for 30 minutes. Two distinct layers of aqueous and organic layers, separated by white coagulated mass of protein were observed after spun. The aqueous phase was transferred into a new 1.5 ml microcentrifuge tube and 900 μ l chilled absolute ethanol of -20⁰C was added. The mixture was incubated at -20⁰C for 30 minutes. After incubation, the mixture was spun at 13,000 rpm, temperature 4⁰C for 10 minutes. The supernatant of absolute ethanol was poured off. The pellet was washed using 1 ml 70% (v/v) ethanol, spun at 13,000 rpm, temperature 4⁰C for 5 minutes. Supernatant containing 70% ethanol was discarded and the pellet was washed with 70% ethanol, spun and supernatant discarded again. The tubes were inverted to drain off excessive fluid and the pellet was subjected to air drying. Completely dried pellet appeared transparent and 100 μ l TE buffer was added to dissolve the pellet. RNase (2 μ l) was then added and the mixture was incubated at temperature 37⁰C for 30 minutes. The extracted DNA was stored at -20 ⁰C for further analysis.

4.2.2 Swab DNA Extraction

Day 1

The prepared swab samples were cut and placed into 1.5 ml labeled tubes. The volume of 580µl dH₂O 320µl 5X Proteinase k Buffer, 20µl Proteinase K, 80µl 20% SDS were added to each tube and mixed gently. The tubes were put in the sponge rock and incubated overnight at 37⁰C water bath.

Day 2

The incubated suspension was cooled to room temperature on the following day. Next, 200 µl of 6M NaCl was added, the mixture was subjected to pulse vortex. The suspension was then transferred equally into 1.5 ml micro centrifuge tubes, with volume of about 350 µl in each tube. Phenol-chloroform (800 µl) was added and the mixture was shaken until milky emulsion was formed. The emulsion was spun at 13,000 rpm, temperature 10⁰C for 30 minutes. Two distinct layers of aqueous and organic layers, separated by white coagulated mass of protein were observed after spun. The aqueous phase was transferred into a new 1.5 ml microcentrifuge tube and 900 µl chilled absolute ethanol of -20⁰C was added. The mixture was incubated at -20⁰C for 30 minutes. After incubation, the mixture was spun at 13,000 rpm, 4⁰C for 10 minutes. The supernatant of absolute ethanol was poured off. The pellet was washed using 1 ml 70% (v/v) ethanol, spun at 13,000 rpm, temperature 4⁰C for 5 minutes. Supernatant containing 70% ethanol was discarded and the pellet was washed with 70% ethanol, spun and supernatant discarded again. The tubes were inverted to drain off excessive

fluid and the pellet was subjected to air-drying. Completely dried pellet appeared transparent and 100 µl TE buffer was added to dissolve the pellet. RNase (2 µl) was then added and the mixture was incubated at temperature 37⁰C for 30 minutes. The extracted DNA was stored at -20⁰C for further analysis.

Material

QIAamp DNA blood mini kit (QIAgen, USA), 1X red cell lysis buffer, 10X proteinase K buffer, 20 mg/ml Proteinase K (Promega, USA), 20% SDS and 400 µl sdH₂O, 6M NaCl, phenol: choloform (1:1, v/v), absolute ethanol (Merck, Germany), 70 % ethanol (Merck, Germany), TE buffer, 20 mg/ml Rnase (Invitrogen, USA).

Equipment

Eppendorf 5415R centrifuge (Eppendorf, Germany), Sigma 3-16 PK centrifuge (Sartorius, Germany), Water bath (Memerct, Germany).

4.3 DNA quantitation and qualification

DNA samples were quantitated by estimation of optical density and agarose gel electrophoresis.

4.3.1 Estimation of optical density (OD)

DNA was diluted 50X by mixing 2 μ l of DNA with 98 μ l of Milli-Q water. Concentration and purity of the DNA was determined by using UV spectrophotometer. Absorbance was measured at 260 nm (A_{260}) and 280 nm (A_{280}). The ratio (A_{260}/A_{280}) of 1.7-2.0 was considered pure genomic DNA.

4.3.2 Agarose gel electrophoresis

Gel electrophoresis was done to confirm the presence of genomic DNA after DNA extraction and to check the DNA quality. Agarose gel (0.8% w/v) was prepared by weighing 0.4 g of agarose powder, dissolved in 50 ml of 1x TBE. The beaker containing agarose powder in TBE was swirled to mix the component and heated in a microwave for 1 minute or until the solution boils. The solution was cooled until 55-60⁰C by putting the beaker of heated solution in cold water. Around 1 μ l of EtBr (10 mg/ml) was added and the solution was poured into a gel casting tray with gel comb set up to form wells; bubbles were avoided. The gel was left for about 30 minutes to let it solidify.

After the gel had solidified, the gel comb was removed and the gel was transferred to the electrophoresis chamber. TBE (1X) was poured to fully submerge the gel. DNA ladder GenRuler™ 1 kb (3 μ l) was loaded into first well of the gel. Appropriate volume of 6X loading dye was mixed with 2 μ l of DNA and loaded into subsequent wells. Electrophoresis

was run at 80 volts for 40 minutes. Then the gel was visualized under UV light using transilluminator.

Materials

Milli-Q® water, 1x TBE, SeaKem® LE Agarose Cambrex (Bio Science, Rockland), EtBr (Sigma Aldrich, USA), GeneRuler™ 6X loading dye (Fermentase, Canada), Lambda DNA/Hind III Marker (Fermentas, Canada).

Equipments

Spectrophotometers (Shimadzu, Japan), Weight balance (Sartorius, Germany), Mini-Sub cell GT electrophoresis system (Biorad, USA), Gel electrophoresis system (Bioneer, Korea).

4.4 Selection of SNPs

Some studies have showed that there is inadequate level of IL10 production in serum of patients in different disease. Various IL10 production have been affected by polymorphisms in the promoter region of the gene such as -1082 G/A and -819 T/C, therefore these SNPs were selected for this study. The details of these SNPs are shown in Table 4.1.

Table 4-4-1: Details of IL-10 SNPs selected

SNP	SNP ID in dbSNP	Location	Classification	Major allele	Minor allele
-1082 (G/A)	rs1800870	Promoter	Transition	A	G
-819 (T/C)	rs1800871	Promoter	Transition	T	C

4.5 Polymerase Chain Reaction (PCR)

This study has been conducted as a case-control experiment on 322 patients with NHL and 290 control subjects. PCR amplification of promoter region containing -1082 (G/A) and -819 (T/C) were carried out in a single tube using ABI thermal cycler. Each reaction contained 30 ng DNA, 1X PCR buffer, 0.25mM dNTPs, 2.5 mM MgCl₂, 0.3 mM of each forward and reverse primer and 0.3 unit of Taq polymerase. The forward and reverse primer nucleotides sequences are shown in Table 4.2 (Eskdale et al., 1999).

Material

10x PCR buffer (Finnzymes, Finland), 10mM dNTP mix (Promega, USA), 50 mM MgCl₂ PCR primers (Research Biolabs, Singapore), Milli-Q® water, Taq polymerase (Finnzymes, Finland), 1x TBE, SeaKem® LE Agarose Cambrex (Bio Science, Rockland), EtBr (Sigma Aldrich, USA), GenRuler™ 6X loading dye (Fermentase, Canada), Gen Ruler™ DNA ladder and ultra-Low Range DNA ladder (Fermentase, Canada).

Equipments

Applied Biosystem 2720 thermal cycler (Applied, Biosystem,USA), Sigma 3-169 k centrifuge (Sartorius,Germany), weight balance (Sartorius,Germany)

Mini-sub Cell GT electrophoresis system (Biorad,USA), Gel electrophoresis system (Bioneer,Korea)

Table 4-4-2: Details of primer sequences, PCR amplicon sizes

Polymorphic Variants	Primer sequences and size (5'→3')	PCR product Size (bp)
IL10-1082819	FW:5'CCAAGACAACACTACTAAGGCTTCTTGAGGA3' (31bp) RV: 5'-AGGTAGTGCTCACCATGA-3' (18bp)	359bp

The reaction mixture for PCR was prepared in Table 4.3.

Table 4-4-3: Reaction mixture for PCR amplification of IL10 -1082 and IL10 -819 SNPs.

Reagent	Stock Concentration	Final Concentration	Volume (μl)
PCR buffer	10X	1X	3.0
dNTPs	10mM	0.25mM	0.75
MgCl ₂	50mM	2.5mM	1.5
Forward primer	10 μl	0.3μM	0.9
Reverse primer	10 μl	0.3μM	0.9
Milli-Q water			19.65
Taq polymerase	3unit/μl	0.9 unit	0.3
DNA Template	15 ng/ μ	30ng	3.0
Total			30.0

PCR condition

The details of PCR cycle conditions were shown in table 4.4.

Table 4-4-4: PCR condition

Step	Temperature	Duration	Number of cycles
Initial denaturation	94 ⁰ C	5 minutes	1
Denaturation	94 ⁰ C	30 seconds	35
Annealing	58 ⁰ C	30 seconds	35
Extension	72 ⁰ C	30 seconds	35
Final extension	72 ⁰ C	7 minutes	2

4.5.1 Gel Electrophoresis for PCR Product

The 3% (w/v) Gels were prepared for electrophoresis of PCR product to confirm successful amplification. 1.8g of agarose powder was weighed and dissolved in 60 ml of 1X TBE. DNA ladder (100bp) was loaded into first well of the gel. Appropriate volume of 6X loading dye was mixed with 4 µl of PCR product and loaded into subsequent wells. Electrophoresis was carried out at 80 volts for 50 minutes.

Material

10x PCR buffer (Finnzymes, Finland), 10mM dNTP mix (Promega, USA), 50 Mm MgCl₂ PCR primers (Research Biolabs, Singapore), Milli-Q® water, Taq polymerase(Finnzymes, Finland), 1x TBE, SeaKem® LE Agarose Cambrex (Bio Science,

Rockland), EtBr (Sigma Aldrich, USA), GenRuler™ 6X loading dye (Fermentase, Canada), Gen Ruler™ DNA ladder and ultra-Low Range DNA ladder (Fermentase, Canada).

Equipments

Thermal cycler (Applied, Biosystem, USA), Sigma 3-169 k centrifuge (Sartorius, Germany), weight balance (Sartorius, Germany), Mini-sub Cell GT electrophoresis system (Biorad, USA), Gel electrophoresis system (Bioneer, Korea).

4.6 Restriction Fragment Length Polymorphism (RFLP) Analysis

The confirmed PCR products containing *IL10-1082* G/A, *IL10-819* G/A fragments, on agarose gel, were digested with BseRI and MslI Restriction enzyme respectively, at 37°C for 16 hours. BseRI is an exonuclease restriction enzyme; its activity is based on incubation of 40 units of enzyme with 1 µg DNA for 16 hours at 37°C in 50 µl of reaction mixture. MslI is an exonuclease restriction enzyme with activity based on incubation of 250 units of enzyme with 1 µg DNA for 16 hours at 37°C in 50 µl of reaction buffer. No enzyme inactivation procedure was needed. The master mix for restriction enzyme was prepared as shown in Table 4.5 and 4.6 Enzymes were purchased from New England Biolabs, Inc (USA).

Table 4-4-5: Master mix for BseRI restriction enzyme

Reagent	Volume(μl)
PCR product	6.0,8.0,10 μ l (depends on Strength of PCR product band)
Buffer4	1.0 μ l
BseRI	0.2
Milli-Q water	2.8
Total	10

Table 4-4-6: Master mix for MslI restriction enzyme

Reagent	Volume(μl)
PCR product	6.0,8.0,10 μ l (depends on strength of PCR product band)
Buffer4	1.0 μ l
MslI	0.2
Milli-Q water	2.8
Total	10

4.6.1 Gel Electrophoresis for RFLP Digestion Products

Appropriate volume of 6X loading dye was mixed with digested products and loaded into the wells. The digested products were resolved by 3% agarose gel electrophoresis with an electric current of 150 volts and 400 ampere to detect genotypes of each individual. The volume of 2 μ l of Generuler DNA ladder 100bp and Generulerultra low range DNA ladder were loaded in the first and last wells respectively as the molecular weight marker.

Electrophoresis was run for 1 hour and 15 minutes. The gels were visualized under UV transition. The details of fragments seen after digestion are shown in Table 4.7.

Table 4-4-7: Digested fragments yield size

	<i>IL10-1082</i>	<i>IL10-819</i>
	AA=356bp GA=356bp, 318bp, 41bp GG=318bp	CC=299bp CT=356bp, 299bp, 60bp TT= 356bp

Material

Milli-Q® (Milli porc,USA) water,NEBuffer4 (New England Biolabs,USA), MslI (New England Biolabs,USA), BseRI(New England Biolabs,USA).TBE buffer, Seakem ® LE Agarose Cambrex (BioScience, Rockland),EtBr (10 mg/mL) (Sigma Aldrich,USA), GenRuler™ 6X loading dye (Fermentase, Canada), Lambda DNA/ HindIII Marker (Fermentase, Canada) and GenRuler™ 100 bp DNA Ladder (Fermentase, Canada).

Equipments

Sigma 3-16Pk centrifuge (Sartorius, Germany), Weight balance (Sartorius, Germany), Mini-Sub Cell GT electrophoresis system (Biorad, USA), Gel electrophoresis system (Bioneer, Korea), Oven incubator (Mettler,Germany)

4.7 DNA Sequencing

Direct sequencing of the PCR products for several samples were carried out by using the reverse primer to validate the genotypes. To ensure minimum noise level that may interfere with interpretation of the sequence result the PCR products were purified from the gels using QIAquick Spin gel purification kit according to the manufacturer's protocol. Gel electrophoresis was done to check the presence of purified DNA. The purified PCR product was sequenced by a commercial sequencing facility (First base Sequencing) to verify the amplicons.

Equipment

Applied Biosystem 2720 thermal cycler (Applied Biosystem, USA), Weight balance (Sartorius, Germany), Mini-sub Cell GT electrophoresis system (Biorad, Korea), Sigma 3-16 k centrifuge (Sartorius, Germany), 3130x Genetic Analyzer (Applied Biosystem, USA), Eppendorf 5415R centrifuge (Eppendorf, Germany)

4.8 Statistical analysis

The observed genotype frequencies and allele frequencies of both SNPs in each three major groups in Malaysia were determined from data obtained. The expected genotype frequencies were calculated from the allele frequencies using the following formula:

$$P^2 + 2pq + q^2 = 1$$

P = frequency of major allele

q = frequency of minor allele

P^2 = frequency of major allele homozygotes

$2pq$ = frequency of heterozygotes

q^2 = frequency of minor allele homozygotes

Genotype distributions frequencies of control population were tested for Hardy-Weinberg equilibrium by Chi-square (χ^2) test for goodness of fit. SNPs association with NHL in every ethnic was tested using Fisher's exact test under allelic, dominant inheritance and recessive inheritance models. Probability values of less than 0.05 were regarded as statistically significant.

$$\chi^2 = \sum (\text{Observed} - \text{Expected})^2 / \text{Expected}$$

Comparisons between different ethnic groups were done using χ^2 2×2 contingency table analysis. Probability values of less than 0.05 indicate significant difference between the groups.

Flowchart of procedures, which carried out in this study, is shown in appendix B.

5 RESULTS

5.1 Study population

NHL cases peripheral blood or buccal swab samples were collected from University of Malaya Medical Center (UMMC) and Ampang Hospital from April 2007 to October 2009. Healthy controls peripheral blood or buccal swab samples were collected around Klang Valley during the same period of time. Buccal swab samples were collected from those participants whose blood samples were not available. In this study 320 and 312 of case samples were used for *IL10*-1082 G/A and *IL10*-819 C/T SNP genotyping respectively. In addition 285 and 284 of control subjects were used for *IL10*-1082 G/A and *IL10*-819 C/T SNP genotyping respectively as well. The collected samples were characterized accordingly. In this study for both control and case groups, the Chinese distribution was more than Malays and Indian.

The ethnicity distribution of cases and controls for each SNP is provided in table 5-1 and 5-2.

Table 5-1: Ethnicity distribution of 320 cases and 285 control subjects for IL10-1082 G/A SNP

Characteristics		Control (n=285) Frequency (100 %)	Case (n=320) Frequency (100 %)
Ethnics	Malays	115 (40.35)	131 (40.93)
	Chinese	144 (50.52)	149 (46.56)
	Indian	26 (9.12)	40 (12.50)

Table 5-2: Ethnicity distribution of 312 cases and 284 control subjects for IL10-819 C/T SNP

Characteristics		Control (n=284) Frequency (100 %)	Case (n=312) Frequency (100 %)
Ethnics	Malays	116 (40.85)	128 (41.02)
	Chinese	142 (50.00)	147 (47.11)
	Indian	26 (9.15)	37 (11.85)

5.2 DNA extraction and quantitation

Genomic DNA was successfully extracted from salivary swab or blood samples of cases and controls including Malay, Chinese and Indian volunteers, by using the conventional phenol-chloroform DNA extraction method. The extracted DNA samples were relatively pure with the A_{260}/A_{280} ratio ranging from 1.6 to 2.0. The yields were satisfactory with a total amount of DNA of about 20-50 μ l from 2 mL blood and about 1 μ g from 3 buccal swab sticks. Electrophoresing of DNA on 0.8% agarose gel is used to define quality of DNA.

Molecular weight band of 23 kb of extracted genomic DNA was achieved from the extraction. Figure 4-1 shows the electrophoresis analysis of the genomic DNA extracted from eight individuals.

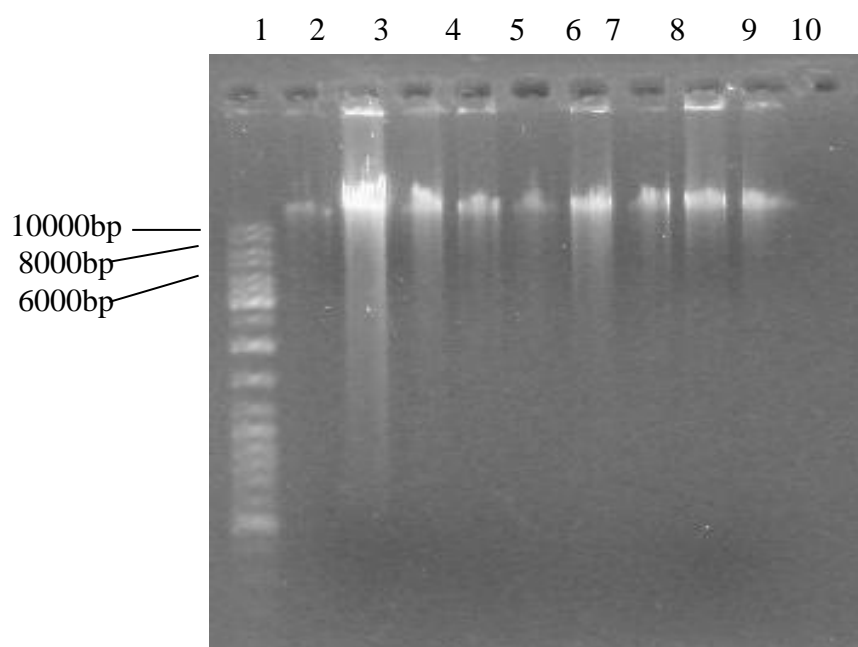


Figure 5-1: Ethidium-bromide stained 0.8% (w/v) agarose gel of extracted genomic DNA

Ethidium-bromide stained 0.8% (w/v) agarose gel of extracted genomic DNA

Lane 1: GenRuler™ 1 kb DNA ladder

Lane 2-10: Different individual genomic DNA samples

5.3 PCR amplification of the promoter region IL10 gene

Two SNPs at *IL10* promoter region (positions -1082 G/A, -819 T/C) were analyzed in the three major ethnic groups in Malaysia. PCR was employed to amplify DNA fragments incorporating these SNP sites. The PCR products were used in RFLP analysis.

A fragment of approximately 359 bp containing both *IL10*-1082 G/A, *IL10*-819 T/C SNPs was successfully amplified. The fragment size obtained corresponded to the expected size Figure 5.2 shows amplicons of *IL10* promoter region.

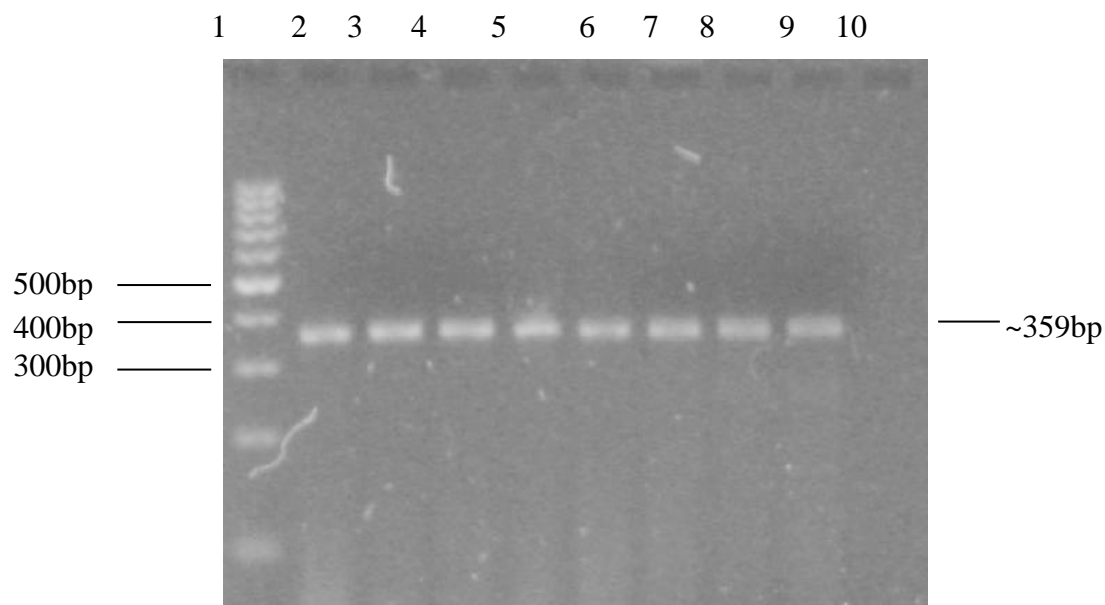


Figure 5-2: PCR amplification of the promoter region of *IL10* gene

Lane 1: 100bp DNA ladder

Lane 2-9: PCR product of the promoter region of *IL10* gene

Lane 10: PCR negative control (DNA blank)

5.4 PCR-RFLP

IL10-1082 G/A

The PCR product was subjected to BseRI Restriction enzyme. After digestion with BseRI, the major allele (A) was resulted in 356bp and 3bp fragments while the minor allele (G) was resulted in fragment of 318bp and 41. However, the smaller fragments of 41bp and

3bp could not be visualized on normal gel electrophoresis. Figure 4-3 shows the PCR-RFLP pattern of the three genotypes of *IL10* 1082 G/A after agarose gel electrophoresis. Individual's genotype was scored based on the PCR-RFLP pattern.

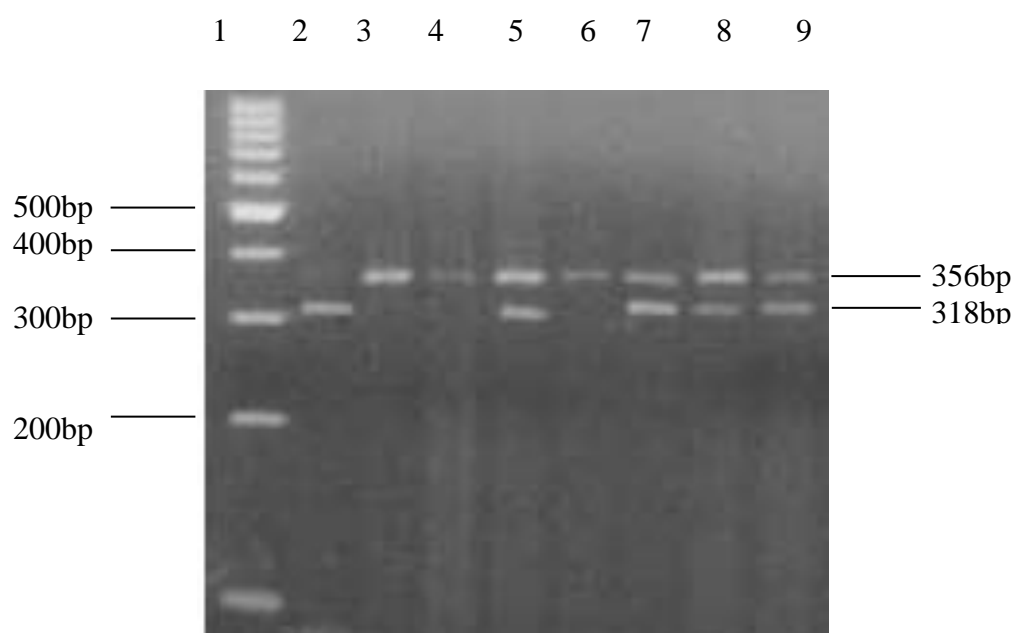


Figure 5-3: Ethidium-bromide stained 3% (w/v) agarose gel of PCR-RFLP pattern of *IL10* 1082 G/A

Lane1:	100bp DNA ladder
Lane2:	318bp fragment (GG genotype)
Lane 3, 4, 6:	356bp fragment(AA genotype)
Lane 5, 7, 8, 9:	318bp, 356bp fragments(GA genotype)

4.3.2 *IL10*-819 T/C

The *IL10*-1082/819 PCR product was subjected to *M*spI Restriction enzyme. After digestion with *M*spI, the minor allele (C) resulted in 299bp and 60bp fragments while the major allele (T) resulted in fragments of 356bp and 3bp. Smaller fragments of 60bp and 3bp could not be seen clearly after agarose gel electrophoresis. Figure 4.3.2 shows ethidium-bromide stained 3% (w/v) agarose gel of PCR-RFLP pattern of the three genotypes of *IL10* 819 T/C. Individual's genotype was scored based on the PCR-RFLP pattern.

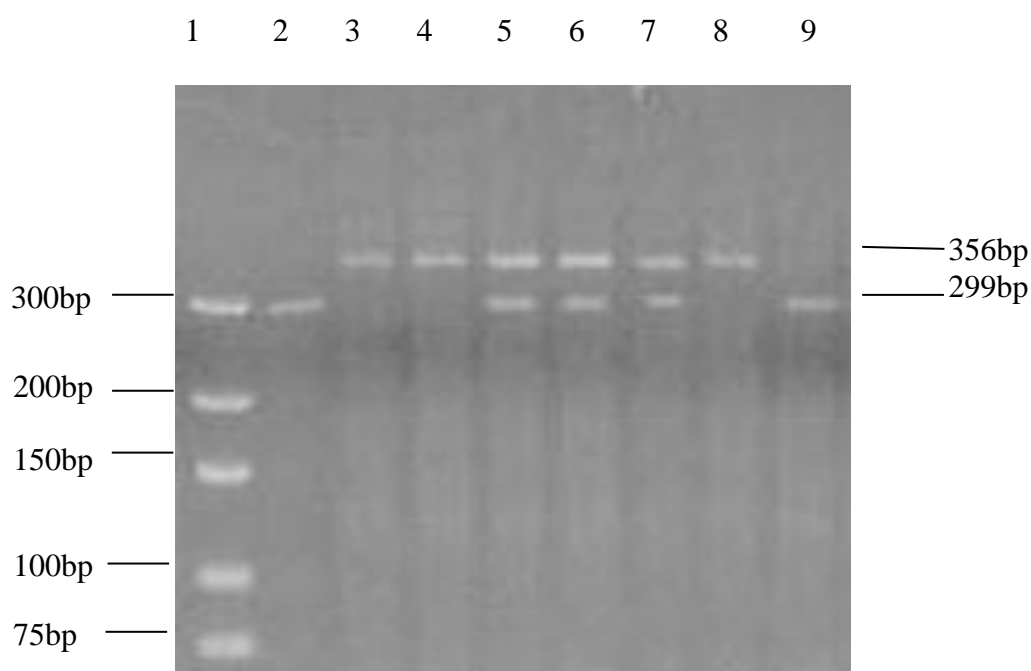


Figure 5-4: Ethidium-bromide stained 3% (w/v) agarose gel of PCR-RFLP pattern of *IL10* 819 T/C

Lane 1: Gene Ruler™ Ultra Low range DNA ladder
 Lane 2,9: 299bp fragment (CC genotype)
 Lane 3, 4, 8: 356bp fragment (TT genotype)
 Lane 5, 6, 7: 299bp and 356bp fragments (CT genotype)

5.5 DNA sequencing

The amplify region generated by the mentioned primers were confirmed by DNA sequencing. The DNA sequences of amplicons were compared to reference sequence in the National Center for Biotechnology Information (NCBI) database by using Basic Local Alignment Search Tool (<http://www.ncbi.nlm.nih.gov/BLAST>).

Figure 5.5, 5.6 and 5.7 indicate the partial sequencing results of purified PCR products of homozygous wild type (TT), homozygous mutant (CC) and heterozygous (CT), polymorphism genotype respectively for *IL10*-819 C/T.

Figure 5.8, 5.9 and 5.10 show partial sequencing result of the purified PCR products as of homozygous wild type (AA), homozygous mutant (GG) and heterozygous (GA), polymorphism genotype respectively for *IL10*-1082 G/A.

The DNA sequences of PCR products containing -1082 and -819 SNPs are shown in appendix D.

G T C T C T A T A A T G T A G T G G (Forward)
30 40
C A G A G A T A T A C A T C A C C
*

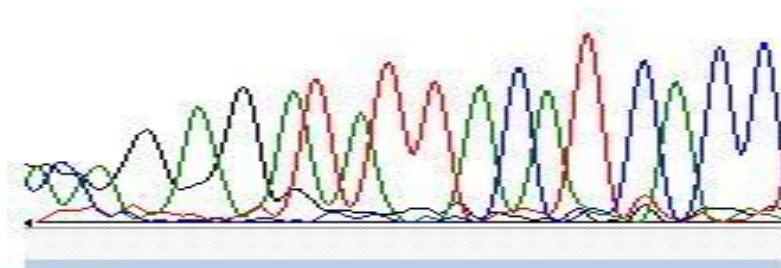


Figure 5-5: The sequencing result of homozygous wild type genotype of *IL10*-819 C/T SNP (TT) on the negative strand on DNA.

The * (bp number 37) indicates the homozygous wild type genotype of the *IL10*819 SNP (TT)

G T C T C T A C A A T G T A G T G G (Forward)
30 40
C A G A G A T G T A C A T C A C C
*

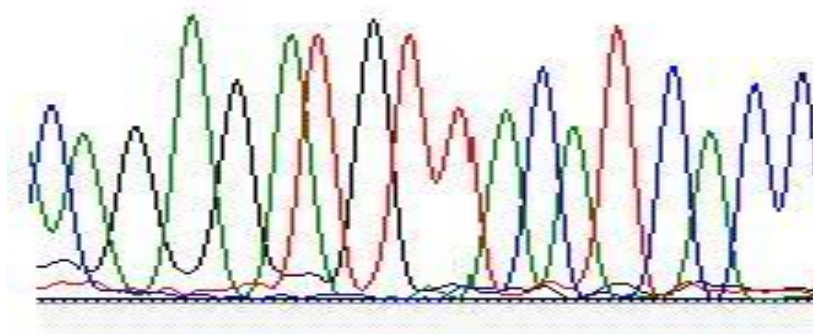


Figure 5-6: The sequencing result of homozygous variant genotype of *IL10*-819 C/T SNP (CC) on the negative strand on DNA.

The * (bp number 33) indicates the homozygous minor allele genotype of the *IL10*-819 C/T SNP (CC).

G T C T C T A T A A T G T A G T G G (Forward)

30 40
CA G AG ATAT TACAT CACC

*

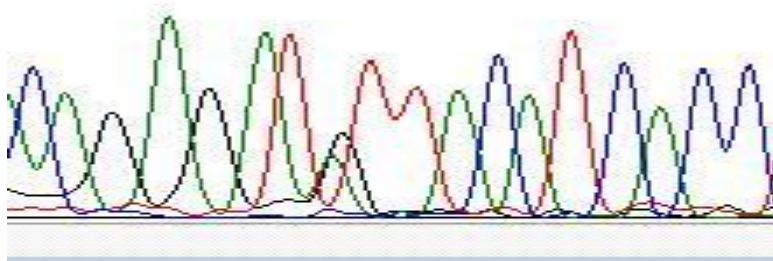


Figure 5-7: The sequencing result of heterozygous genotype of *IL10*-819 C/T SNP (CT) on the negative strand of DNA.

The * (bp number 37) indicates the heterozygous genotype of the *IL10*819 C/T SNP (CT).

G A T G A A G G G G A A G G (Forward)

300
CTACTTCCCTTCC

*

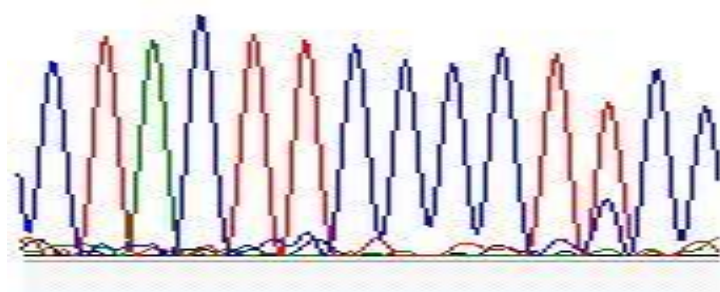


Figure 5-8: The sequencing result of homozygous wild type genotype of *IL10*-1082 G/A SNP (AA)

The * (bp number 304) indicates the homozygous wild type genotype of the *IL10*-1082 G/A SNP (AA).

G A T G A A G G G G A G G (Forward)

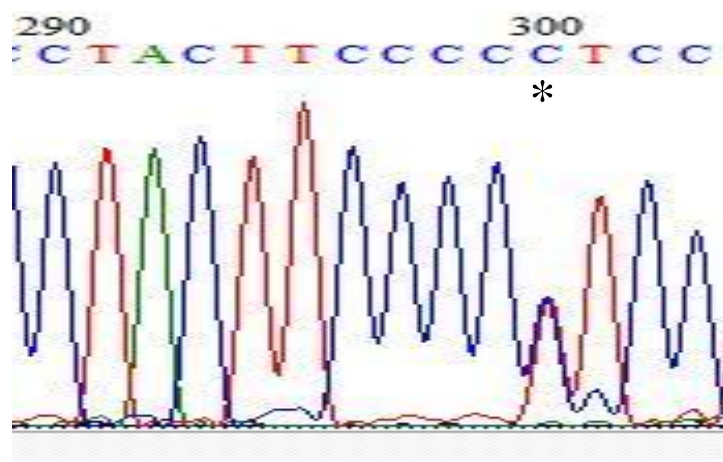


Figure 5-9: The sequencing result of heterozygous genotype of *IL101082* SNP G/A on the negative strand of DNA.

The * (bp number 300) indicates the heterozygous genotype of the *IL101082* SNP (GA).

G A T G A A G G G G A G G (Forward)

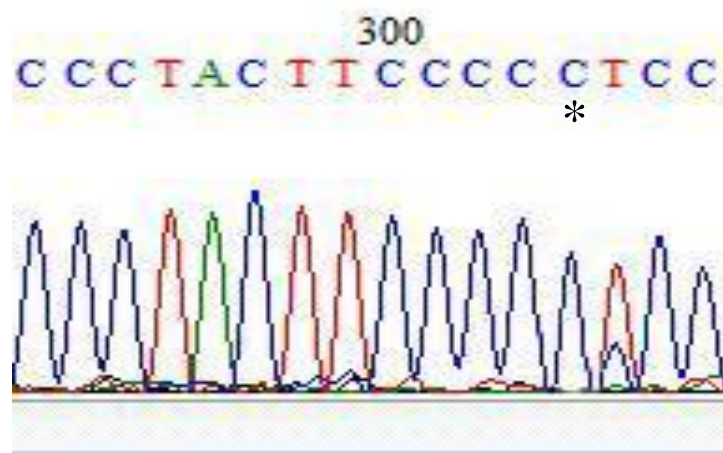


Figure 5-10: The sequencing result of homozygous variant genotype of *IL10-1082* G/A SNP (GG) on the negative strand of DNA.

The * (bp number 304) indicates the homozygous variant genotype of the *IL10-1082* G/A SNP (GG).

5.6 Statistical analyses

5.6.1 Chi-square (χ^2) test of Hardy-Weinberg equilibrium (HWE)

Genotype and allele frequencies of both SNPs in each of the three major groups were calculated. Hardy-Weinberg equilibrium (HWE) of the genotype distribution in control group was examined. The distribution of *IL10*-1082 G/A and *IL10*-819 T/C genotypes of all control population fit the Hardy-Weinberg equilibrium law.

5.6.2 Association of *IL10*-1082G/A with NHL

IL10-1082 G/A association with NHL in every ethnic group was examined by using Fisher's exact test under allelic, dominant inheritance and recessive inheritance models. Association of *IL10*-1082 G/A SNP with NHL in different ethnics (Malaysian Chinese, Malaysian Malay and Malaysian Indian) are shown in Table 5.2, 5.3 and 5.4.

5.6.2.1 Chinese

In the Chinese population, calculating p value by using Fisher's exact ($p > 0.05$) test showed that the association is not significant. Therefore this SNP is not likely to be associated with increasing risk of NHL.

5.6.2.2 Malays

In the Malay population, calculating P value ($p>0.05$) by using Fisher's exact test showed that the association is not significant. Therefore this SNP is not likely to be associated with increasing risk of NHL.

5.6.2.3 Indian

In the Indian population, calculating P value ($p>0.05$) by using Fisher's exact test showed that the association is not significant. Therefore this SNP is not likely to be associated with increasing risk of NHL.

5.6.3 Association of IL10-819 C/T with NHL

IL10-819 C/T association with NHL in every ethnic group also was examined by using Fisher's exact test under allelic, dominant inheritance and recessive inheritance models. Association of *IL10*-819 C/T SNP with NHL in different ethnics are shown in Table 5.2, 5.3 and 5.4.

5.6.3.1 Chinese

In Chinese population, calculating p value ($p>0.05$) by using Fisher's exact test, showed that the association between this SNP and NHL is not significant. Therefore this SNP is not associated with increasing risk of NHL.

5.6.3.2 Malays

In Malays population, calculating P value by using Fisher's exact test under allelic model showed that association between this SNP and NHL is significant ($p = 0.023$) For recessive inheritance P value was 0.029 that express significant association between SNP and NHL. Therefore this SNP is associated with the risk of NHL.

5.6.3.3 Indian

In Indian population, calculating P value by using Fisher's exact test, showed that the association between this SNP and NHL is not significant. Therefore this SNP is not likely to be associated with increasing risk of NHL.

Table 5-5-3: Association of *IL10* 1082 G/A and -819 C/T SNPs with NHL in Chinese population

Gene	SNP No	Cases				Controls					P value			Odds ratio	95% confidence interval
		WT	HET	VAR	MAF	WT	HET	VAR	MAF	HWE (P value)	Allelic model	Dominant model	Recessive Model		
IL10	1082 A>G	129	20	--	0.0671	129	15	--	0.0520	0.50	0.48	0.47	1.0	0.16	0.097-0.260
IL10	819 T>C	69	56	22	0.340	77	52	13	0.274	0.33	0.10	0.24	0.15	1.36	0.954-1.941

WT: Homozygous wild type; **HET:** Heterozygous; **VAR:** Homozygous Variant; **MAF:** Minor allele frequency G allele for -1082 and Allele for -819; **HWE:** Hardy-Weinberg equilibrium

P value computed by Fisher's exact test 2×2 table

p < 0.05, significant difference between cases and healthy control

Table 5-5-4: Association of *IL10* 1082 and 819 SNPs with NHL in Malays population

Gene	SNP No	Cases				Controls					P value			Odds ratio	95% confidence interval
		WT	HET	VAR	MAF	WT	HET	VAR	MAF	HWE (P value)	Allelic model	Dominant model	Recessive model		
IL10	1082 A>G	107	23	1	0.0954	98	16	1	0.078	0.70	0.52	0.49	1.0	0.22	0.134-0.343
IL10	819 T>C	56	54	18	0.351	63	47	6	0.254	0.46	0.023	0.12	0.029	1.59	1.074-2.351

WT: Homozygous wild type; **HET:** Heterozygous; **VAR:** Homozygous Variant; **MAF:** Minor allele frequency G allele for -1082 and C allele for -819; **HWE:** Hardy-Weinberg equilibrium

P value computed by Fisher's exact test 2×2 table

p < 0.05, significant difference between cases and healthy control

Table 5-5-5: Association of *IL10* 1082 and 819 SNPs with NHL in Indian population

	SNP No	Cases				Controls					P value			Odds ratio	95% confidence interval
		WT	HET	VAR	MAF	WT	HET	VAR	MAF	HWE (P value)	Allelic model	Dominant model	Recessive model		
IL10	1082 A>G	24	15	1	0.212	22	4	-	0.0769	0.67	0.16	0.05	1.0	3.24	1.02-10.25
IL10	819 T>C	20	13	4	0.283	12	14	-	0.269	0.060	1.0	0.61	0.13	1.08	0.486-2.379

WT: Homozygous wild type; **HET:** Heterozygous; **VAR:** Homozygous Variant; **MAF:** Minor allele frequency G allele for -1082 and C allele for -819; **HWE:** Hardy-Weinberg equilibrium

P value computed by Fisher's exact test 2×2 table

p < 0.05, significant difference between cases and healthy control

6 DISCUSSION

Non-Hodgkin lymphoma (NHL) is a malignant disease of the immune system with the most rapidly increasing incidence in many countries. The inequality in frequency of NHL patients among different populations is notable. Since Malaysia is a country with multi-ethnic community, it is of interest to consider variations in the incidence of NHL among different populations. The causes of NHL still remain unidentified, but genetic variations have been suggested as increased risk factor of NHL (Howell and Rose-Zerilli, 2007)

Many studies have been done on gene association for different kinds of human diseases. There are several findings in specifically NHL showed the effectiveness of the gene susceptibility as a high risk factor (Domingo-Domenech et al., 2007). In addition NHL risk is associated with conditions that alter the immune system; thus it is logical to investigate inherited genetic variation of genes coding for cytokines such as interleukin 10. In this study we managed to investigate the relationship between *IL10*-1082 G/A and *IL10*-819 T/C SNPs of *IL10* and NHL in three major ethnic groups. (Howell and Rose-Zerilli, 2007).

6.1 Genomic DNA extraction and quantification

The conventional phenol-chloroform extraction method was used to extract genomic DNA because it gave satisfactory yields of good quality DNA and was cheap. However, a major disadvantage of this method is use of highly toxic reagents such as phenol and chloroform. If the phenol is not completely removed; it will interfere with the quantification of DNA by using UV absorbance because phenol has a very high destruction coefficient at 260 nm. Whereas the yield is sometimes slightly lower, the major advantage of using DNA extraction kit is that the phenol and chloroform are avoided and it is fast and easy.

6.2 Genotyping method

In this study we use PCR –RFLP genotyping method to detect DNA sequence changes in *IL10*-819C/T and *IL10* -1082A/G. We confirmed the genotypes for the three PCR-RFLP patterns by direct DNA sequencing. Our result demonstrated that this technique enabled a clear distinction between the three genotypes. This indicates that PCR-RFLP genotyping is a reliable method for the genotyping of these two SNPs.

6.3 Statistical analysis

All genotype distribution of healthy control populations for both SNPs is in agreement with the Hardy-Weinberg equilibrium (HWE). Agreement of *IL10*-819 C/T and *IL10*-1082 G/A with HWE law also was also reported in Iranian and Caucasian populations (Fife et al., 2006, Azarpira et al., 2010).

In this study, we found that *IL10*-1082G/A SNP is not associated with the increasing risk of NHL since the *p* value is not less than 0.05. Several association studies on the *IL10*-1082A/G and cancers has shown similar result and some reported conflicting results. Sugitomoto et al. (2007) reported that there is no association between *IL10*-1082 G/A SNP and risk of gastric cancer and Hodgkin's disease (Howell and Rose-Zerilli, 2007).

Published findings have noted the association of the *IL10*-1082 AA genotype with susceptibility, advanced stage of disease and greater tumor thickness of cutaneous malignant melanoma and susceptibility to aggressive NHL as well as prostate cancer and renal cell carcinoma. (Howell and Rose-Zerilli, 2007).

In addition Pablo Sáenz López et al. (2009) introduced allele G as a risk allele for renal cancer. They also discovered that AG heterozygosity status is the main risk factor relative to locally advanced or more advanced tumor disease (Sáenz López, 2009), while Yao et al. (2008) also reported gene promoter *IL10*-1082A/G polymorphism, is significantly associated with the risk of oral cancer (Yao et al., 2008b).

The frequency of (-1082G) allele was found to be higher in patients with DLBCL as compared with control subjects. They also showed patients with the (-1082G) allele have higher complete remission rate and were more susceptible as compared with patients carrying the (-1082AA) genotype (Domingo-Domenech et al., 2007) . Purdue et al. (2006) also reported that the -1082G is associated with an increased risk of DLBCL (Purdue et al., 2006).

It has been suggested that -1081G/A polymorphism might affect the translation process of IL10. This polymorphism lies within an Ets (E-twenty-six specific)-like recognition site and may affect binding of this transcriptional factor and thus vary transcriptional activation. The -1082A allele has been correlated with low IL10 production while -1082G has been reported to be associated with high level expression of IL10 (Bialecka et al., 2007).

Among several studies, which have been done on *IL10*-819 C/T and cancer, no association has been reported. Pablo Sáenz López et al. (2009) reported that there is no association between -819 C/T SNP and risk of renal cancer (Sáenz López, 2009). No association of this SNP has been found with prostate cancer, cervical cancer, myelodysplasia, acute myeloid leukemia and gastric cancer as well as cutaneous malignant melanoma (Howell and Rose-Zerilli, 2007). According to study by Purdue et al. (2007) there is no association between this SNP and risk of NHL, FL and DLBCL (Purdue et al., 2006). However, we demonstrated in our study that -819T/C is associated with increasing risk of NHL in the Malay population. *P* value for allelic and recessive models are 0.0235 and 0.0297 respectively, which indicate difference between cases and controls, is considered to be

statistically significant; but there is no association of this SNP and NHL in the Chinese and Indian population in Malaysia.

Referring to our result on significant association of -819C/T and NHL it is only observed in the Malay population. Furthermore lack of association between this SNP and cancers in previous studies suggest that replication study with larger samples is needed to confirm our result. According to previous studies *IL10*-818 C allele (recessive allele) is linked with higher secretion of IL10. On the other hand in Malays association was observed under recessive model. Therefore it can be concluded that this allele and also higher level of IL10 is associated with NHL.

Limitation of this study:

Several limitations were identified in this study. Firstly the small sample size which might not represent the true population and had effect on reliability of the findings. Our Indian population also is not big enough. To verify the findings, a larger sample size is needed. Secondly this study lacks related phenotypic and functional assay, which limits the study inquiry in to the functional consequence of this variance. Thirdly the relationship between level of IL10 expression in patients with different alleles of these two SNPs did not study. and different type of *in vitro* or *in vivo* functional assays need to be carried out to investigate the functional of implications of the -819 C/T SNP in the future.

7 CONCLUSION

Based on the findings of this study -819T/C genetic polymorphism is likely to be associated with risk NHL in Malay. But -1082A/G is unlikely to be associated with the risk of malignant NHL in Chinese, Malay and Indian ethnic groups in Malaysia .